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March 1974

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β-Diethylaminoethyl-diphenylpropylacetate (SKF-525-A), a surface-active agent, inhibits sickling of erythrocytes from individuals with homozygous sickle cell anemia (SS cells) *in vitro*. Sickling was prevented when SS cells were incubated in 3×10^{-4} M SKF-525-A, washed free of excess drug, and then deoxygenated in humidified nitrogen atmosphere. Polymerization of sickle hemoglobin and submembrane vesicles could be demonstrated in these cells by electron microscopy. At 3×10^{-4} M SKF-525-A approximately 10% of the cells lysed. Incubation of pretreated cells at 37°C without added oxygen for 24 hours did not produce significant additional lysis. Irreversibly sickled cells were unaffected by drug treatment. Preliminary studies suggest that the oxygen affinity of sickle hemoglobin in the intact cell is not altered by SKF-525-A. It is hypothesized that inhibition of sickling is the result of drug-induced alterations of the cell membrane.

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SUMMARY

β-Diethylaminoethyl-diphenylpropylacetate (SKF-525-A), a surface-active agent, inhibits sickling of erythrocytes from individuals with homozygous sickle cell anemia (SS cells) *in vitro*. Sickling was prevented when SS cells were incubated in 3×10^{-4} M SKF-525-A, washed free of excess drug, and then deoxygenated in humidified nitrogen atmosphere. Polymerization of sickle hemoglobin and submembrane vesicles could be demonstrated in these cells by electron microscopy. At 3×10^{-4} M SKF-525-A approximately 10% of the cells lysed. Incubation of pretreated cells at 37°C without added oxygen for 24 hours did not produce significant additional lysis. Irreversibly sickled cells were unaffected by drug treatment. Preliminary studies suggest that the oxygen affinity of sickle hemoglobin in the intact cell is not altered by SKF-525-A. It is hypothesized that inhibition of sickling is the result of drug-induced alterations of the cell membrane.

PREFACE

The work described in this report was authorized under Task 1T061102B71A02, Life Sciences Basic Research in Support of Materiel–Chemical. This work was started in October 1971 and completed in June 1972.

Acknowledgment

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I. INTRODUCTION.

Hemoglobin molecules may bind tightly to the erythrocyte membrane and actually contribute to its structure.[1,2] Abnormalities produced in the interaction of intracellular hemoglobin molecules can thus alter membrane structure and function. Deoxygenation and polymerization of intracellular sickle hemoglobin induces characteristic changes in red cell shape which are reversible with reoxygenation and depolymerization. Jensen and Lessin[2] demonstrated that the unsickling process in some cells may be associated with apposition and fusion of the inner surfaces of the cell membrane. They postulate this membrane interaction leads to fragmentation and formation of rigid "irreversibly" sickled erythrocytes.

Irreversibly sickled erythrocytes cause increased blood viscosity. Their mechanical fragility is increased and they are susceptible to erythrophagocytosis. The morbidity of sickle cell anemia should thus be lessened by the prevention of formation of irreversibly sickled cells. Prevention of polymerization of deoxygenated sickle hemoglobin would accomplish this as would interference with subsequent membrane interaction. The present paper discusses the effects of β-diethylaminoethyl-diphenylpropylacetate (SKF-525-A), a surface-active agent, on cells containing sickle hemoglobin.

II. MATERIALS AND METHODS.

Reagents and chemicals were obtained as follows: β-diethylaminoethyl-diphenylpropylacetate from Smith, Kline, and French (Philadelphia, Pennsylvania); trypan blue from Allied Chemical Corporation (New York, New York); Hanks' balanced saline solution without bicarbonate (HBSS), pH 6.8, from Grand Island Biological Company (Grand Island, New York); Difco Noble agar from Difco Laboratories (Detroit, Michigan); stoppered tubes containing 7.5 mg of disodium Edetate (EDTA) from Becton, Dickinson, and Company (Rutherford, New Jersey); and components for Dulbecco phosphate buffered saline (PBS), pH 7.2, from J. T. Baker Chemical Co. (Phillipsburg, New York). The saline solution used in washing and resuspending cells was 0.9% sodium chloride.

Five milliliters of blood was drawn by syringe, added aseptically to 7.5 mg of disodium EDTA, stoppered, mixed gently, and refrigerated at 4°C. Studies were carried out on samples stored for less than 96 hours. Blood was obtained from healthy, nonanemic Caucasian volunteers and individuals known to have homozygous sickle cell anemia, sickle C disease, and sickle trait. Erythrocytes from these individuals will be referred to as AA cells, SS cells, SC cells, and AS cells respectively. AA, SS, SC, and AS cells were obtained from whole blood samples by centrifugation at 750 x g in a Model PR-2 International centrifuge at 4°C for 10 minutes. The buffy coat was removed, and the erythrocytes were washed three times with 5 ml of saline and resuspended in either 10 ml of saline or HBSS. Leukocyte contamination of these suspensions was negligible. Erythrocyte concentrations were enumerated by a Model 6301 laser cytograf (Biophysics Systems, Inc., Mahopac, New York).[3,4]

A. Effect of SKF-525-A on Sickling.

Washed erythrocytes were suspended to a final concentration of 10^9 cells/ml in 5 ml of HBSS or PBS containing 3×10^{-4} M SKF-525-A. Similar cell suspensions without drug were also

prepared. After incubation for 15 minutes at room temperature, the cell suspensions were centrifuged. The erythrocytes were washed twice with HBSS or PBS and resuspended in 5 ml of HBSS or PBS in 50-ml-capacity screwcap tubes (Falcon Plastics, Los Angeles, California). Each cap was pierced with two 19-gauge needles and sealed airtight. One of the needles was connected to a plastic catheter which terminated below the surface of the cell suspension. One hundred percent nitrogen gas was bubbled through distilled water into the cell suspension through this tubing for 15 minutes to provide a humidified nitrogen atmosphere.* Neutral buffered formalin was then rapidly added to the cell suspension through a stopcock. A "Y"-shaped connecting tube was affixed to two or more of the tubes so that control and drug-treated red cells were deoxygenated simultaneously. The 10% neutral-buffered-formalin fixed-cell preparations were immobilized in 0.8% agar containing 0.4% trypan blue for dark field phase microscopy. SS cells from each patient were placed in both HBSS and PBS and each suspension was incubated with and without SKF-525-A for phase and electron microscopic studies.

B. Effect of SKF-525-A on Cell Lysis.

Washed red cells (AA, SS, SC, and AS cells) were suspended in 10 ml of HBSS and the red cell concentration was determined. SKF-525-A was added to paired 1-ml aliquot samples in concentrations of 1×10^{-4}, 3×10^{-4}, and 5×10^{-4} M SKF-525-A. The paired samples with drug and a pair of 1-ml aliquot samples without drug were incubated at room temperature for 10 minutes and centrifuged, and the supernatant was aspirated. The cells were washed twice with 1 ml of saline, centrifuged, and brought to a final volume of 10 ml with saline. The cell concentrations of these samples were determined. The percent hemolysis was obtained by dividing the average cell count at each drug concentration by the average cell count of the samples incubated without drug, multiplied by 100.

Four samples of both AA cells and SS cells were prepared and incubated with the above three concentrations of SKF-525-A and without drug for 10 minutes at room temperature, washed twice in 1-ml aliquots of saline, and resuspended in 1 ml of saline. Mineral oil was layered on the top of the suspensions and the preparations were incubated for 24 hours at 37°C. Aseptic handling of the samples was carried out. The mineral oil was aspirated and the cells were diluted to 10 ml with saline and counted. The percent cell lysis was determined as above.

The effect of duration of incubation of a given concentration of SKF-525-A on cell lysis was examined. SS cells were incubated as paired samples without drug and with 3×10^{-4} M SKF-525-A for 10, 30, and 60 minutes at room temperature.

C. Effect of SKF-525-A on Hemoglobin Structure and Red Cell Membrane.

The effect of SKF-525-A on hemoglobin structure and red cell membrane was determined by electron microscopic studies. SS cells were prepared in both HBSS and PBS as outlined above with the exception that fixation of cells was obtained by the addition of glutaraldehyde instead of neutral buffered formalin. After immersion in 2.5% glutaraldehyde in 0.15 M cacodylate buffer for 2 hours at 0°C, the cells were washed three times with 0.1 M cacodylate buffer containing 5% sucrose. Cell pellets were postfixed in Caufield's fixative[5] for 30 minutes at 0°C, rapidly dehydrated through ascending concentrations of ethanol, and embedded in epoxy resin.[6]

*Cerami, A. Personal communication. 1973.

Ultrathin sections prepared with an LKB Ultratome® were counterstained with aqueous solutions of uranyl acetate followed by lead citrate. Grids were viewed in an RCA, EMU-4 electron microscope at an accelerating voltage of 50 kilovolts. All electron micrographs were prepared by Fotorite® automatic processing.

D. Effect of SKF-525-A on Oxygen Affinity.

Washed SS cells were incubated for 15 minutes at room temperature with and without 3×10^{-4} M SKF-525-A in PBS, washed twice with saline, and resuspended to comparable concentrations in the patient's own plasma. Oxygen saturation, pO_2, pCO_2, and pH were measured in a co-oximeter combined with a Model 313 blood gas analyzer (Instrumentation Laboratory, Inc., Watertown, Massachusetts) and the P_{50} was determined.[7]

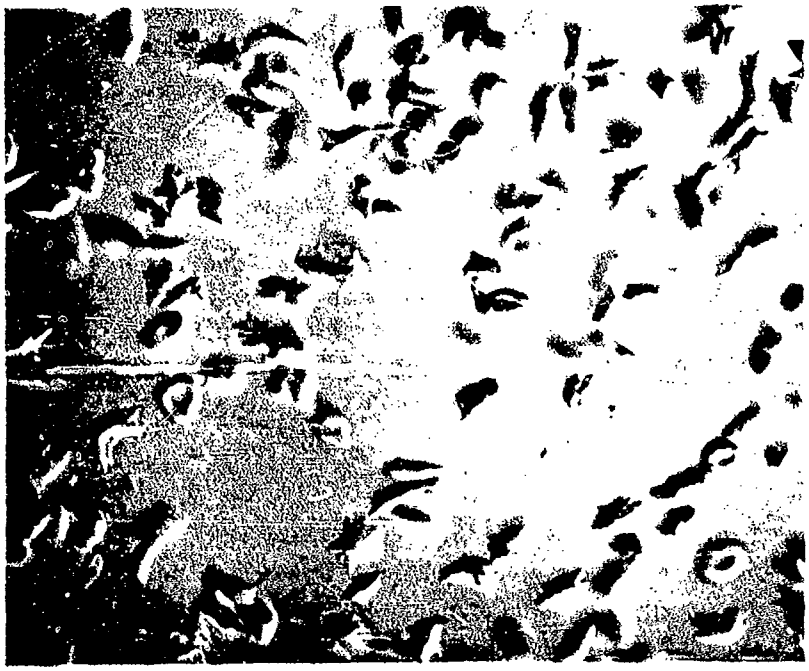
III. RESULTS.

Sickling of SS cells is inhibited by pretreatment of the cells with 3×10^{-4} M SKF-525-A for 15 minutes as demonstrated in figure 1. Deoxygenation of pretreated SS cells results in predominantly cup-shaped, ovoid, and spherocytic erythrocytes (figure 1,B) rather than sickled forms (figure 1,A). Sickling is not prevented if the concentration of SKF-525-A is 1×10^{-4} M or less. Inhibition of sickling occurred whether the SS cells were pretreated with 3×10^{-4} M SKF-525-A in phosphate buffered saline, pH 7.2 and osmolarity 307 milliosmols, or in Hanks' solution, pH 6.8 and osmolarity 286 milliosmols. Treatment with SKF-525-A did not alter the shape of SS cells after they had become irreversibly sickled.

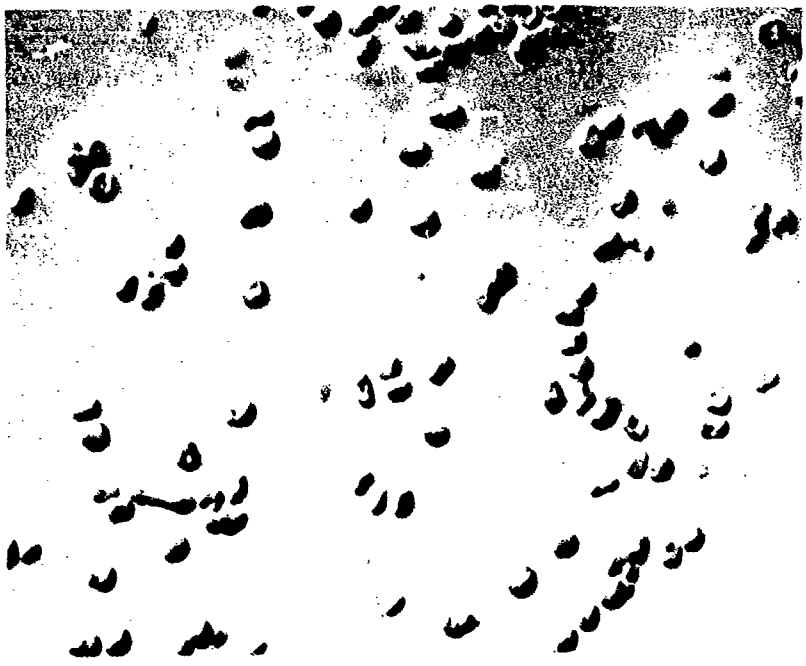
SKF-525 A induces cell lysis. The amount of cell lysis increases with increasing concentrations of drug (figure 2). The presence of sickle hemoglobin within the red cells appears to offer protection against drug-induced lysis. At 3×10^{-4} M SKF-525-A, a concentration of drug sufficient to inhibit sickling, only 10% of SS cells lysed, as compared to 50% of AA cells. The differences in degree of lysis for AA cells and SS cells at 3×10^{-4} M and 5×10^{-4} M SKF-525-A are significant ($P<0.05$). AS and SC cells containing one-third to one-half as much sickle hemoglobin respond in a manner similar to SS cells. There is no statistical difference in degree of lysis of AS, SC, and SS cells at any one of the drug concentrations.

The effect of drug on lysis of SS cells appeared to be maximal at 10 minutes. The percent lysis did not increase when the cells were incubated with SKF-525-A for 30 or 60 minutes. The amount of lysis was not increased with either AA or SS cells after incubation for 24 hours under mineral oil after a 10-minute incubation with SKF-525-A.

Deoxygenation by exposure to humidified nitrogen atmosphere produces aggregates of polymerized hemoglobin characteristic of sickled cells (figure 3,A). In the SKF-525-A-treated cell (figure 3,B), the aggregates of polymerized hemoglobin appear shorter and more numerous than in untreated sickled cells. In untreated sickled cells, the hemoglobin aggregates appear to run within the plane of the axis of distortion (figure 3,A), whereas, in the SKF-525-A-treated cells, consistently fewer strands of polymerized hemoglobin appear to be in the plane of distortion of the cell (figure 3,B). Submembrane vesicles are apparent in drug-treated sickled erythrocytes but not in untreated cells. The vesicles become more numerous and larger as the concentration of SKF-525-A is increased. The alterations in the electron microscopic appearance are similar whether PBS or HBSS was used.



A. SS cells incubated without SKF-525-A.



B. SS cells from same patient pretreated with 3×10^{-4} M SKF-525-A.

Figure 1. Photomicrographs (Nomarsky Optics) of Formalin-Fixed SS Cells after Exposure for 15 Minutes to Humidified Nitrogen Atmosphere (Magnification, 450 X)

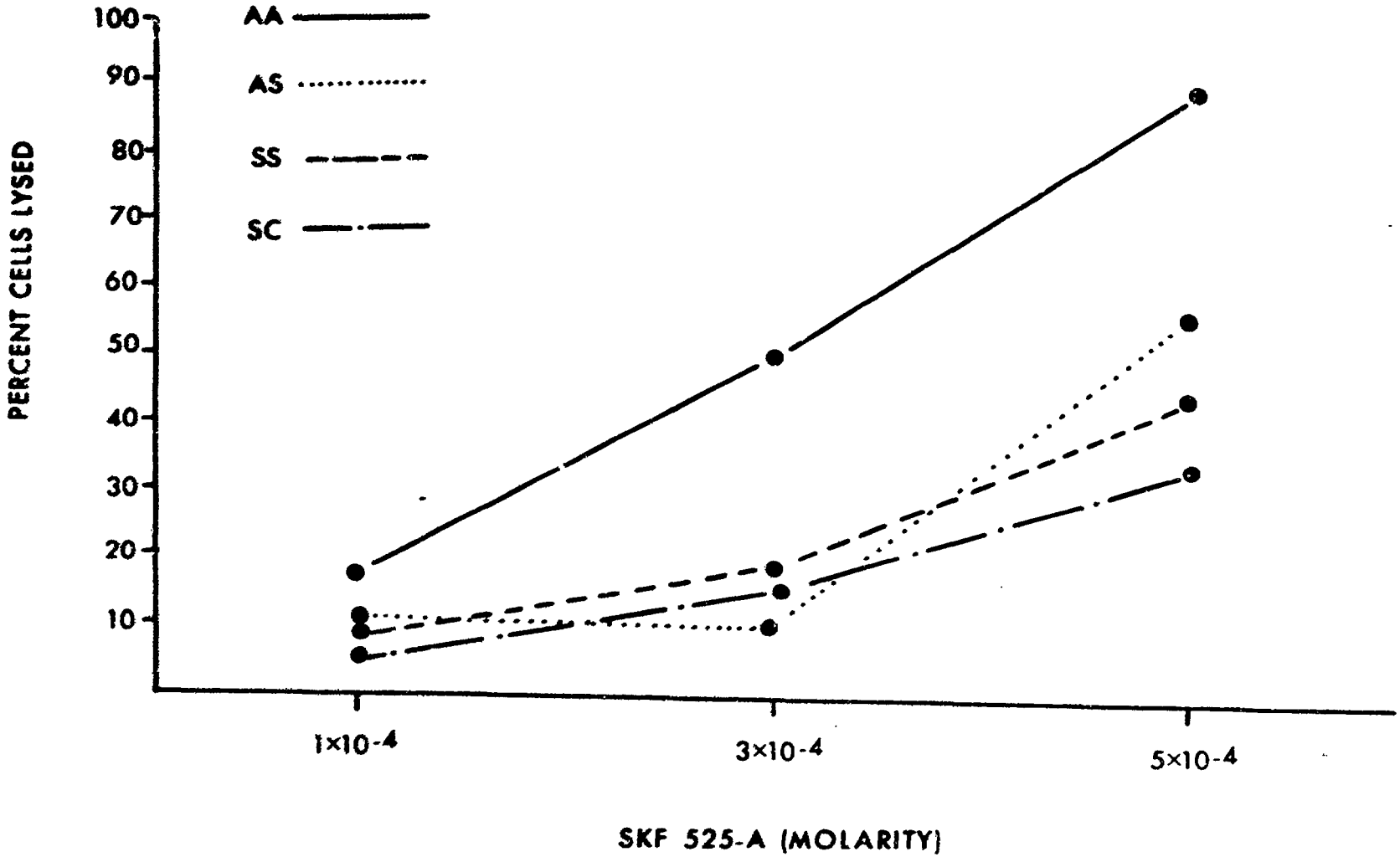


Figure 2. Percent Lysis of AA Cells (4), AS Cells (4), SS Cells (5), and SC Cells (3) in the Presence of Different Concentrations of SKF-525-A

The numbers within the parentheses indicate the number of persons studied.

A. Cells without treatment with SKF-525-A.

B. Cells treated with 3×10^{-4} M SKF-525-A before exposure to nitrogen atmosphere (magnification, 18,000 X).

Figure 3. Electron Micrographs of SS Cells after Nitrogen-Induced Anoxia

After incubation for 24 hours under mineral oil at 37°C, no significant polymerization of hemoglobin is seen in either drug-treated or untreated SS cells. Submembrane vesicles are apparent in the SKF-525-A-treated cells but not in the untreated-cell preparations.

IV. DISCUSSION.

Phenothiazines[8] and corticosteroids[9] have been shown to inhibit sickling *in vitro*, probably on the basis of membrane stabilization.[9] SKF-525-A is a surface-active agent and stabilizes the erythrocyte membrane.[10,11] The present study was thus undertaken to evaluate its effects on sickling *in vitro*.

The effect of phenothiazines and steroids on cell membrane stability is dependent on drug concentration. Lysis occurs at higher concentrations and stabilization occurs at lower concentrations. The molar range of effective concentrations for membrane stabilization is narrow.[12,13] SKF-525-A adsorbs to the erythrocyte membrane[10,11] as do the phenothiazines.[12] However, in contrast to the phenothiazines, SKF-525-A has been shown to stabilize erythrocyte membranes against the effects of hypotonic saline solutions over the wide range of 10^{-9} to 10^{-4} M.[11]

Exposure to 3×10^{-4} M SKF-525-A for 15 minutes inhibited sickling of SS cells but did not alter the appearance of so-called "irreversibly" sickled cells. Only a small amount of cell lysis occurred. Sickling was not inhibited at 1×10^{-4} M SKF-525-A, a concentration known to stabilize erythrocyte membranes.[11] Inhibition of sickling was not affected by variations of pH and osmolarity used in these studies. Since the cells had been washed free of unadsorbed drug, the inhibitory effect on sickling was not primarily osmotic. However, secondary drug-induced alterations of cell permeability cannot be excluded.

Other investigators[1,2,14] have indicated an interaction between polymerized sickle hemoglobin and the red cell membrane which differs from the relationship found in AA cells. SKF-525-A-induced membrane changes are suggested by the findings of significant cell lysis of drug-treated AA cells. This lytic effect is altered by the presence of sickle hemoglobin. Phase microscopic studies show that sickling has been prevented in SKF-525-A-treated deoxygenated SS cells although the electron microscopic studies demonstrate polymerized sickle hemoglobin and submembrane vesicles within the cells (figure 3, A and B). The presence of submembrane vesicles in the absence of polymerized sickle hemoglobin aggregates in SS cells kept for 24 hours under oil further supports a membrane mediated effect.

Preliminary studies suggest that oxygen affinity is not appreciably altered by treatment with SKF-525-A, indicating that denaturation of sickle hemoglobin may not be a factor in the drug effect (figure 4). Denaturation of cell membrane protein is not ruled out as a possibility. Irreversibly sickled cells are unaffected by the drug presumably because the cell membrane is already damaged. Resolution of the problem of membrane damage will require further investigation.

In conclusion, SKF-525-A has been shown to inhibit sickling *in vitro*. The effect is dose dependent in a narrow range of molar concentrations. On the basis of the data obtained, inhibition of sickling by the drug appears to be related to the effect of SKF-525-A on cell membrane. The molecular basis of the drug effect remains to be identified. Clinical usefulness of SKF-525-A in the prevention of sickling *in vivo* is dependent on low drug toxicity[15] and persistence of the demonstrated drug-induced membrane changes. SKF-525-A-induced inhibition of sickling could thus be the result of either drug therapy or extracorporeal treatment and reinfusion of drug-treated SS cells.

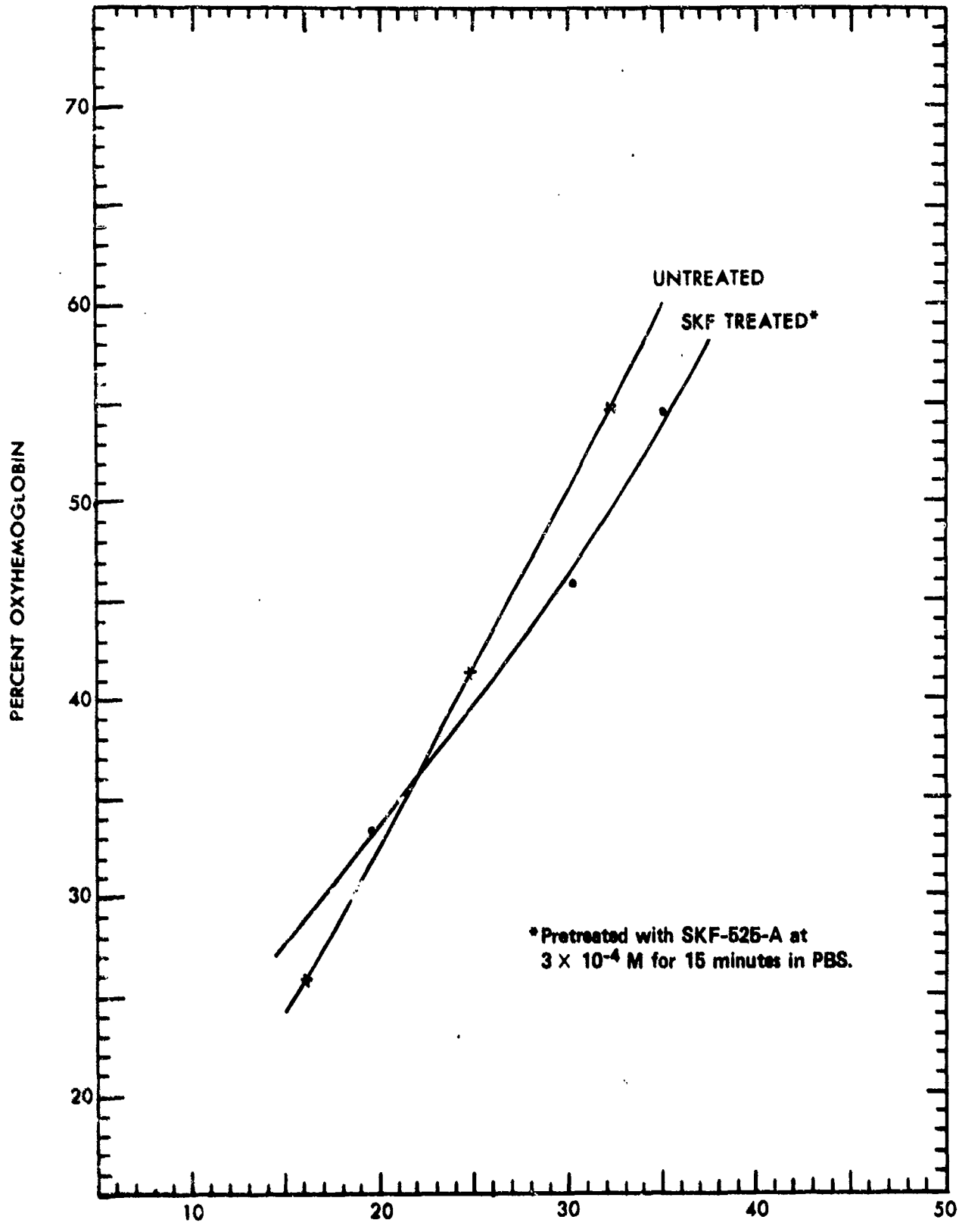


Figure 4. Oxygen Affinity ($P_{50}O_2$) of SS Cells

LITERATURE CITED

1. Bertles, J. F., and Dobler, J. Reversible and Irreversible Sickling: a Distinction by Electron Microscopy. Blood *33*, 884 (1969).

2. Jensen, W. N., and Lessin, L. G. Membrane Alterations Associated with Hemoglobinopathies. Semin. Hematol. *7*, 409 (1970).

3. Melamed, M. R., Kamentsky, L. A., and Boyse, E. A. Cytotoxic Test Automation: a Live-Dead Cell Differential Counter. Science *163*, 285 (1969).

4. Drake, W. P., Ungraro, P. C., and Mardiney, M. R. Formalin Fixed Cell Preparations as Standard for Use in the Automated Trypan Blue Cytotoxic Assay. Transplantation *14*, 127 (1972).

5. Caufield, J. B. Effects of Varying the Vehicle for OSO_4 in Tissue Fixation. J. Biophys. Biochem. Cytol. *3*, 827 (1957).

6. Luft, J. H. Improvements in Epoxy Resin Embedding Methods. J. Biophys. Biochem. Cytol. *9*, 409 (1961).

7. De Furia, F. G., Miller, D. R., Cerami, A., and Manning, J. M. The Effects of Cyanate in Vitro on Red Blood Cell Metabolism and Function in Sickle Cell Anemia. J. Clin. Invest. *51*, 566 (1972).

8. Lewis, R. A., and Gyange, F. N. Inhibition of Sickling by Phenothiazines: Comparisons of Derivatives. Arch. Int. Pharmacodyn. Ther. *153*, 158 (1965).

9. Isaacs, W. A., and Hayhoe, F.G.J. Steroid Hormones in Sickle Cell Disease. Nature *215*, 1139 (1967).

10. Florence, A. T. Some Physico-Chemical Properties of SKF-525-A in Aqueous Solution. J. Pharm. Pharmacol. *22*, 1 (1970).

11. Lee, I. P., Yamamura, H. I., and Dixon, R. L. The Effects of β-Diethylamino-ethyl-Diphenylpropylacetate (SKF 525-A) on Biological Membranes—I. SKF 525-A-Induced Stabilization of Human Erythrocytes. Biochem. Pharmacol. *17*, 1671 (1968).

12. Seeman, P., and Weinstein, J. I. Erythrocyte Membrane Stabilization by Tranquilizers and Antihistamines. Biochem. Pharmacol. *15*, 1737 (1966).

13. Seeman, P. Membrane Stabilization by Drugs: Tranquilizers, Steroids, and Anesthetics. Int. Rev. Neurobiol. *9*, 145 (1966).

14. Dobler, J., and Bertles, J. F. The Physical State of Hemoglobin in Sickle-Cell Anemia Erythrocytes in Vivo. J. Exp. Med. *127*, 711 (1968).

15. Barnes, C. D., and Eltherington, L. G. Drug Dosage in Laboratory Animals. A Handbook. University of California Press, Los Angeles, California. p 223. 1964.